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Suppression of Keratinocyte Growth and Differentiation by Transforming Growth Factor β1 Involves Multiple Signaling Pathways

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Transforming growth factor β1 treatment of keratinocytes results in a suppression of differentiation, an induction of extracellular matrix production, and a suppression of growth. In this study we utilized markers specific for each of these functions to explore the signaling pathways involved in mediating these transforming-growth-factor-β1-induced activities. In the first instance, we found that the induction of extracellular matrix production (characterized by 3TP-Lux reporter activity) was induced in both keratinocytes and a keratinocyte-derived carcinoma cell line, SCC25, in a dose-dependent manner. Furthermore, transforming growth factor β1 also suppressed the differentiation-specific marker gene, transglutaminase type 1, in both keratinocytes and SCC25 cells. In contrast, transforming growth factor β1 inhibited proliferation of keratinocytes but did not cause growth inhibition in the SCC25 cells. Transforming-growth-factor-β1-induced growth inhibition of keratinocytes was characterized by decreases in DNA synthesis, accumulation of hypophosphorylated Rb, and the inhibition of the E2F:Rb-responsive promoter, cdc2, and an induction of the p21 promoter. When the negative regulator of transforming growth factor β1 signaling, SMAD7, was overexpressed in keratinocytes it could prevent transforming-growth-factor-β1-induced activation of the 3TP-Lux and the p21 promoter. SMAD7 could also prevent the suppression of the transglutaminase type 1 by transforming growth factor β1 but it could not inhibit the repression of the cdc2 promoter. These data indicate that the induction of 3TP-Lux and p21 and the suppression of transglutaminase type 1 are mediated by a different proximate signaling pathway to that regulating the suppression of the cdc2 gene. Combined, these data indicate that the regulation of transforming growth factor β1 actions are complex and involve multiple signaling pathways. ***Key words: SMAD7/squamous carcinoma/squamous differentiation. J Invest Dermatol 116:266–274, 2001***

There is a growing body of evidence to suggest that the maintenance of epidermal growth and differentiation is controlled, *in vivo*, by the combined action of positive and negative factors. In the instance of the epidermis, the likely candidates to fulfill these roles are the mitogen, transforming growth factor α (TGF-α), and the tumor suppressor, transforming growth factor β1 (TGF-β1). Recent experimental evidence indicates that overexpression of either cytokine results in perturbations to epidermal growth and differentiation. For instance, transgenic mice overexpressing TGF-α in skin are characterized by epidermal hyperplasia and a propensity for skin tumor formation (Vassar and Fuchs, 1991; Dominey *et al*, 1993; Wang *et al*, 1995). On the other hand, overexpression of TGF-β1 in the skin of transgenic mice results in the inhibition of epidermal proliferation and ultimately death (Cui *et al*, 1995). Furthermore, transgenic mice in which a dominant negative mutant of the type II TGF-β receptor was expressed specifically in the skin were characterized by epidermal hyperplasia (Wang *et al*, 1997). Thus, both TGF-α and TGF-β appear to be key regulators responsible for maintaining the proliferation rate of keratinocytes *in vivo*.

Earlier studies with cultured keratinocytes have shown that TGF-β1 (i) suppresses keratinocyte proliferation (Pietenpol *et al*, 1990a, b; Saunders and Jetten, 1994; Alexandrow and Moses, 1995); (ii) induces extracellular matrix (ECM) production (Vollberg *et al*, 1991); and (iii) suppresses differentiation (Saunders and Jetten, 1994). This combination of effects would seem reasonable as this would allow the epidermal basal cells considerable "reserve" proliferative potential to respond to injury whilst actively maintaining an undifferentiated phenotype. As basal keratinocytes are responsible, in part, for the production of their basement membrane, the effects on the ECM would appear self-evident. The same rationale may be applied to the role of TGF-β1 in the wounded epidermis.

Given that TGF-β1 treatment of keratinocytes results in three different biologic outcomes (growth suppression, differentiation suppression, and ECM induction) it is of interest to define the signaling pathway(s) that mediate these seemingly disparate effects. Until recently, the events linking the activation of the plasma-

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Abbreviations: FACS, fluorescence-activated cell sorting; PAI-1, plasminogen activator inhibitor 1 gene; TG1, transglutaminase type 1; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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membrane-bound TGF-β receptor to nuclear transcriptional events, and hence suppression of growth and differentiation, were unclear. Recent studies identifying the SMAD family of signal transducers, however, have allowed a reasonably clear picture of how TGF-β1 receptor activation can initiate transcriptional activation of TGF-β1-responsive genes in other cell systems (Massague, 1996; Alevizopoulos and Mermod, 1997; Heldin *et al*, 1997; Wrana and Pawson, 1997). A current model for TGF-β1 signal transduction is that receptor occupation leads to phosphorylation and activation of the type I receptor by the type II receptor (Bassing *et al*, 1994; ten Dijke *et al*, 1994; Wieser *et al*, 1995; Feng and Derynck, 1997). This results in phosphorylation of either SMAD2 or SMAD3 and subsequent binding of the phosphorylated SMAD protein to a cytoplasmic SMAD4 protein (Zhang *et al*, 1996). This complex is then translocated into the nucleus where it associates with DNA binding factors such as FAST-1 and *trans*-activates genes with TGF-β1 response elements (Massague, 1996). A recent report suggests that TGF-β1-mediated *trans*-activation may also be mediated directly by SMAD3/SMAD4 heterodimers (Dennier *et al*, 1998). One of the activated genes may be an inhibitory SMAD protein such as SMAD6 or SMAD7 (Imamura *et al*, 1997; Nakao *et al*, 1997; Afrakhte *et al*, 1998). SMAD6 or SMAD7 operate as negative regulators of the signal transduction pathway by inhibiting receptor-mediated activation of the SMAD2 or SMAD3 signal transducers. This signaling pathway has been shown to be conserved in a number of cell systems and is definitively associated with the activation of transcription *via* TGF-β-responsive elements in the plasminogen activator inhibitor 1 gene (PAI-1; Hayashi *et al*, 1997; Imamura *et al*, 1997; Nakao *et al*, 1997; Whitman, 1997). The role of the SMAD proteins, if any, however, in the regulation of other TGF-β1-mediated effects such as suppression of growth or differentiation in keratinocytes is less clear. The importance of determining the events by which TGF-β1 mediates its effects on keratinocytes takes on greater significance in light of the data implicating defects in TGF-β1 regulation in various tumors. In fact, the original cloning of the human SMAD4 gene was as a tumor suppressor gene involved in pancreatic cancer (Hahn *et al*, 1996). Since then various tumor cell lines and patient tumors have been examined for mutations within the TGF-β1 signaling pathway (Park *et al*, 1994; Antar *et al*, 1995; Reiss *et al*, 1997; De *et al*, 1998; Lu *et al*, 1998). Of the SMAD proteins studied to date only SMAD2 and SMAD4 have been found to carry mutations in a subset of tumors (Riggins *et al*, 1997).

With the exception of a recent study with activin signaling in B cells (Ishisaki *et al*, 1998), there have been no definitive studies linking the SMAD signaling pathway with either TGF-β1-mediated suppression of growth or squamous differentiation. In fact, there is considerable evidence to suggest that the signaling pathways that mediate the effects of TGF-β on growth and TGF-β-mediated *trans*-activation are functionally independent. For instance, earlier studies by Chen *et al* (1993) reported that a truncated type II TGF-β receptor was associated with a loss of TGF-β1-mediated growth inhibition whereas PAI-1 *trans*-activation was maintained. Furthermore, it has been reported that the loss of TGF-β1-mediated growth inhibition, but not PAI-1 *trans*-activation, in lesional vascular endothelial cells was attributable to a loss of type II TGF-β receptor expression (McCaffrey *et al*, 1995). Whether different TGF-β signaling pathways are responsible for the control of growth inhibition and PAI-1 induction, however, remains unclear (Cui *et al*, 1995; Massague, 1996; Heldin *et al*, 1997).

In this study we specifically looked at whether (i) the suppression of proliferation, (ii) the suppression of differentiation, and (iii) the induction of ECM by TGF-β1 utilized similar signaling pathways. In order to do this we used well-established markers such as the phosphorylation status of Rb (Dyson, 1998), cdc2 promoter activity (Dahler *et al*, 1998; Dicker *et al*, in press), and p21 promoter activity (Datto *et al*, 1995; Li *et al*, 1998; Moustakas and Kardassis, 1998) as our markers of proliferation, the expression of transglutaminase type 1 (TG 1) as our marker of differentiation (Saunders *et al*, 1993b; Saunders and Jetten, 1994; Dicker *et al*, in press), and the activation of the 3TP-Lux reporter (derived from the PAI-1 promoter) as our marker of ECM production (Wrana *et al*, 1992). We report that the suppression of differentiation and the induction of PAI-1 are mediated by an SMAD7-inhibitable signaling pathway(s) whereas TGF-β1-mediated growth inhibition involves multiple signaling pathways, one of which is SMAD7 insensitive (cdc2 suppression) and another of which is SMAD7 sensitive (p21 induction).

EXPERIMENTAL PROCEDURES

Cell culture and DNA synthesis assays Human epidermal keratinocytes were isolated and cultured from neonatal foreskins following circumcision (Saunders *et al*, 1993a; Jones *et al*, 1997). The keratinocyte-derived sqamous carcinoma cell line, SCC25, was originally isolated from a squamous carcinoma of the tongue and was purchased from the American Type Culture Collection (Rockville, MD) and cultured in Ham's F12:Dulbecco's modified Eagle's medium (1:1, vol:vol) containing 10% serum and supplemented with penicillin/streptomycin (10,000 U per ml; Life Technologies, Sydney, Australia; Jones *et al*, 1997; Dicker *et al*, in press). When required, DNA synthesis was estimated by the incorporation of [^{3}H] methyl thymidine into cellular DNA as described previously (Saunders *et al*, 1993a, 1999a). TGF-β1 and 12-O-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma (Sydney, Australia) and interferon-γ (IFN-γ) was purchased from Life Technologies.

Protein isolation and western blotting Cells, grown in 75 cm^2 flasks, were trypsinized and rinsed with cold phosphate-buffered saline (PBS) and collected by centrifugation (200*g* for 2 min). Cells were immediately resuspended in 6 × protein sample buffer (Saunders *et al*, 1999b), boiled for 3 min, and stored at −70°C until required. When required, 20 μg of protein was then boiled for 3 min and electrophoresed on a 7.5% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). The proteins were transferred to a PVDF membrane and blocked for 6 h at room temperature in 3% skim milk powder (wt/vol) in PBST (PBS + 0.05% Tween-20, vol/vol). A rabbit polyclonal antibody against human Rb (sc-50; Santa Cruz Biotechnology, Brisbane, Australia) or a rabbit polyclonal antibody against human cdc2 (Ab-1; Oncogene Science, Melbourne, Australia) was then incubated with the blot overnight at 4°C in 3% skim milk powder in PBST. Blots were washed in PBST and incubated with horseradish peroxidase conjugated antirabbit monoclonal antibody (Selenus, Sydney, Australia) for 2 h at room temperature in 3% skim milk powder in PBST. Blots were then washed thoroughly with PBST and the specific proteins were visualized by ECL detection (Amersham, Sydney, Australia).

Transfection and reporter assays The 3TP-Lux reporter gene contains three TGF-β1-responsive elements from the PAI-1 promoter linked to the firefly luciferase gene (Wrana *et al*, 1992). A 949 bp fragment of the human cdc2 promoter directing expression of a CAT reporter gene (cdc2-CAT) has been described previously (Dalton, 1992; Dahler *et al*, 1998). The cdc2-CAT construct contains an E2F:Rb-responsive element at −125 bp (Dalton, 1992). A 291 bp fragment of the human p21 promoter (Zeng *et al*, 1997) containing a TGF-β1-responsive element (Datto *et al*, 1995; Moustakas and Kardassis, 1998) driving the expression of a luciferase reporter gene has been described previously. A squamous differentiation-specific 2.9 kb fragment of the rabbit transglutaminase type I promoter coupled to a luciferase reporter gene has also been described (Saunders *et al*, 1993b; Medvedev *et al*, 1999). The β-actin CAT reporter gene (Saunders *et al*, 1993b) or a β-actin luciferase reporter gene (Dahler *et al*, 1998) were used to normalize for transfection efficiency. Transient transfections of human epidermal keratinocytes and SCC25 cells were performed on 30% confluent cultures and assayed for reporter activity 48 h post-transfection. For human epidermal keratinocytes, 10 cm^2 wells were rinsed once with minimal serum-free medium (SFM; Life Technologies) and transfected with 1 μg reporter (e.g., 3TP-Lux, p21, or cdc2-CAT) plus 0.3 μg β-actin CAT/luciferase and 4 μl lipofectamine reagent (Life Technologies). The lipofectamine and the individual plasmids were prepared separately in 100 μl of minimal SFM each, mixed together, and then left for 30 min at room temperature. These solutions were mixed with 0.8 ml minimal SFM per transfection. The diluted complexes were added to the rinsed cells for 5 h at 37°C at which time the medium was replaced with complete SFM. In some instances cotransfections with an expression plasmid coding for SMAD7 (Nakao *et al*, 1997) were carried out in human epidermal keratinocytes. These were performed in a similar manner to that described

above except that 0.3 μg of CMV SMAD7 or the control vector pCDNA3 (InVitrogen, Melbourne, Australia) were included in the transfection.

SCC25 cells were transfected by the calcium phosphate method. Cells were fed with 1 ml complete 1:1 medium (Jones *et al*, 1997) immediately prior to transfection. For each well, 2 μg 3TP-Lux and 2 μg β-actin CAT were diluted to 100 μl with sterile water and 25 μl of 2.5 M $CaCl_2$ was added. The DNA-$CaCl_2$ mix was added dropwise to 125 μl 2 × HEPES-buffered saline (pH 7.05) being bubbled through by a mechanical pipette and the solution was briefly vortexed. The precipitate was allowed to form for 30 min at room temperature and was overlaid onto the fed cells. Cells were incubated for 8 h and then shocked for 3 min at room temperature with 15% glycerol (vol/vol), followed by three PBS washes. Cells were refed with 2 ml complete 1:1 medium and incubated for 48 h.

Chloramphenicol acetyltransferase and luciferase reporter assays have been described previously (Saunders *et al*, 1993b; Dahler *et al*, 1998; Medvedev *et al*, 1999). In instances where dose-response curves were analyzed, data were subjected to nonlinear least squares regression analysis and fitted to the equation $e = (E_{max} \times Ec_{50})/(L + Ec_{50})$ using the software analysis program Prism (GraphPad, U.S.A.), where e is the percentage induction above untreated cells, E_{max} is the maximal effect, L is the TGF-β1 concentration (ng per ml), and Ec_{50} is the concentration of TGF-β1 at which half maximal response was observed.

RNA isolation, northern blotting, and reverse transcriptase polymerase chain reaction (RT-PCR) The isolation of total cellular RNA and the detection by northern blotting has been described previously (Saunders *et al*, 1993b; Saunders and Jetten, 1994). Generation of a probe for TG 1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been described previously (Saunders *et al*, 1993b). Probes for the detection of the type I or type II TGF-β receptors were generated by PCR amplification using previously described primers (Guo and Kyprianou, 1998). A probe for the detection of SMAD7 was generated from a BamH1/EcoR1 restriction fragment from the corresponding expression plasmid. To normalize for loading inequalities northern blots were also probed with GAPDH (Saunders *et al*, 1993b). In experiments addressing TG 1 mRNA half-life, the blots were analyzed by phosphorimage analysis as described earlier (Saunders *et al*, 1998; 1999a). In all other instances the transcript expression was visualized by exposure to Kodak XAR5 film (Kodak, Sydney, Australia).

The analysis of mRNA expression by RT-PCR has been described (Jones *et al*, 1997; Saunders *et al*, 1998, 1999a). RT-PCR analysis was used to examine the expression of the differentiation-specific gene TG 1 and the housekeeping gene actin in human epidermal keratinocytes following fluorescence-activated cell sorting (FACS) of transfected cell populations.

FACS analysis FACS was used to purify trasnfected cells out of keratinocyte cultures using an EPICS Elite ESP flow cytometer (Coulter, Hiateah, FL). FACS analysis was performed in experiments determining the ability of SMAD7 to inhibit the suppression of differentiation (characterized by TG 1 mRNA expression).

In studies examining the SMAD7 sensitivity of the suppression of TG 1 by TGF-β1, human epidermal keratinocyte cultures (1 × 25 cm^2 flask per condition) were treated with TPA (50 ng per ml) for 16 h at which time they were cotransfected with either the pCDNA3 vector (2.6 μg) or the SMAD7 expression vector (2.6 μg) plus an expression vector coding for green fluorescent protein (0.8 μg, CMV-GFP; Clontech, Sydney, Australia). Five hours after transfection with lipofectamine, the cells were refed with medium containing TPA (50 ng per ml) or TPA (50 ng per ml) + TGF-β1 (20 ng per ml). Twenty-four hours later the human epidermal keratinocytes were trypsinized and passed through 75 μm gauze into FACS tubes. Human epidermal keratinocytes expressing GFP were then sorted and collected into Eppendorf tubes and centrifuged (10 s at 10,000*g*). The cell pellet consisting of between 4000 and 10,000 cells was subjected to RNA isolation and the equivalent of 10^3 cells were used for RT-PCR analysis of TG 1 and actin mRNA expression. RT-PCR analysis was performed under linear conditions with respect to cycle number to assure quantitative determination of gene expression.

RESULTS

SCC25 cells possess an intact SMAD-dependent signaling pathway TGF-β1 caused a dose-dependent decrease in human epidermal keratinocyte proliferation ($Ec_{50} = 3.2 \pm 1.3$ ng per ml; **Fig 1*A***). Consistent with an earlier report (Shipley *et al*, 1986), a keratinocyte-derived carcinoma cell line, SCC25, was resistant to TGF-β1-induced growth inhibition up to a concentration of 30 ng per ml (**Fig 1*B***). As loss of TGF-β1 responsiveness in other carcinoma cell lines has been previously shown to be associated with defects in TGF-β1 receptor/SMAD signaling (Park *et al*, 1994; Antar *et al*, 1995; Reiss *et al*, 1997), we examined the ability of the human epidermal keratinocytes and SCC25 cells to activate the TGF-β1-responsive elements contained in the 3TP-Lux reporter construct. The 3TP-Lux reporter gene contains three TGF-β1-responsive elements from the promoter of the ECM protein, PAI-1, linked to the firefly luciferase gene (Wrana *et al*, 1992). Both human epidermal keratinocytes and SCC25 cells were able to induce 3TP-Lux activity following TGF-β1 treatment (**Fig 1*C***). In fact, the SCC25 cells (**Fig 1*B***) appeared more sensitive

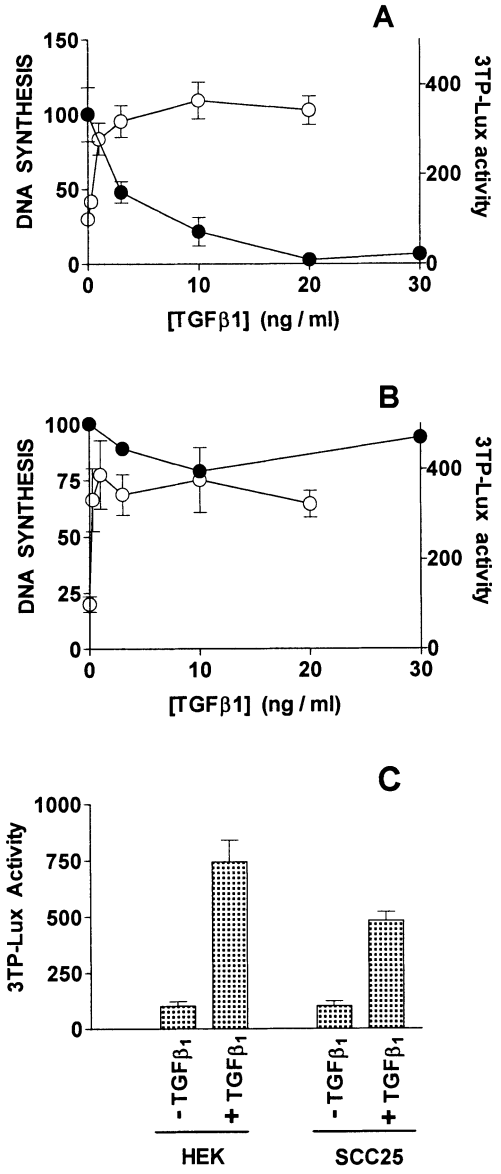


Figure 1. Human epidermal keratinocytes and SCC25 cells activate 3TP-Lux in response to TGF-β1. Proliferating human epidermal keratinocytes (*A*) or SCC25 cells (*B*) were incubated with varying concentrations of TGF-β1 for 48 h, following which DNA synthesis (●) was estimated by a thymidine incorporation assay (dpm incorporated per μg protein) or 3TP-Lux activity was assayed (○). Data are presented as a percentage of the untreated cells. Data points represent mean ± SEM. (*C*) TGF-β1-mediated *trans*-activation was measured in human epidermal keratinocytes or SCC25 cells. Cells were transfected with reporter constructs as described in *Experimental Procedures* and were then treated for 48 h with (+TGF-β1; 20 ng per ml) or without (–TGF-β1) TGF-β1 following which luciferase activity was estimated. Data represent mean ± SEM of at least three determinations.

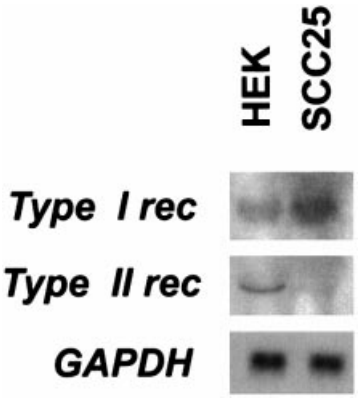


Figure 2. SCC25 cells express low levels of TGF-β type II receptor mRNA. Total RNA (30 μg) from proliferating human epidermal keratinocytes or SCC25 cells was subjected to northern blotting and probed for mRNA expression levels of the type I (Type I rec) and type II (Type II rec) TGF-β receptor. The expression of GAPDH was also estimated to allow for loading inequalities.

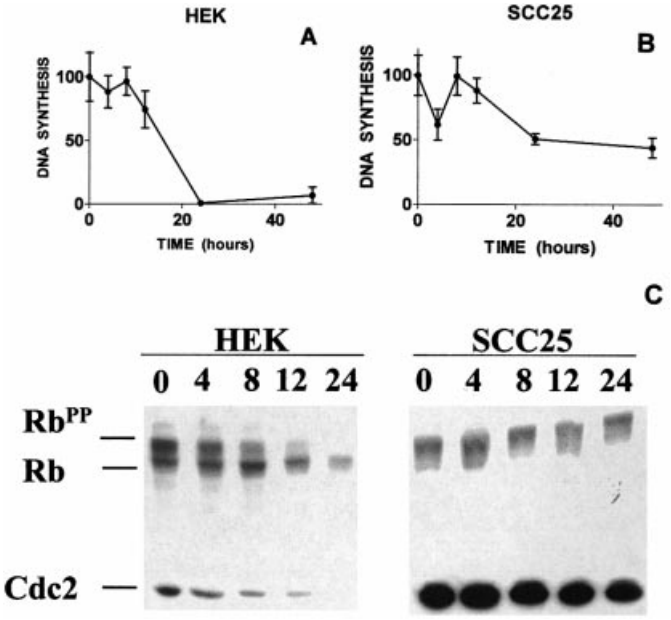


Figure 3. SCC25 cells are resistant to TGF-β1-induced Rb hypophosphorylation and cdc2 downregulation. (*A*) Proliferating human epidermal keratinocytes and SCC25 cells were treated with TGF-β1 (20 ng per ml) for varying times at which point DNA synthesis was estimated by thymidine incorporation (dpm per μg protein). Data are the mean ± SEM of at least three determinations expressed as a percentage of the untreated sample. (*B*) Human epidermal keratinocytes and SCC25 cells were harvested for determination of Rb phosphorylation status and cdc2 protein levels following treatment with TGF-β1 (20 ng per ml) for varying times. In this instance, proteins were isolated and 20 μg were run on a 7.5% SDS-PAGE gel and blotted to PVDF membrane. The hypophosphorylated form (Rb) and the hyperphosphorylated form (Rb^{PP}) were detected by their different mobilities following immunoblotting. The expression levels of the cdc2 protein are also shown.

to TGF-β1 [$E_{max} = 356 \pm 32$; $Ec_{50} = 0.01 \pm 0.06$ ng per ml; degrees of freedom (df) = 16] than were the human epidermal keratinocytes (**Fig 1*A***; $E_{max} = 365 \pm 26$, $Ec_{50} = 0.41 \pm 0.16$ ng per ml; df = 16) with respect to 3TP-Lux activation. This finding was significant as it indicated that both human epidermal keratinocytes and SCC25 cells had an intact receptor/SMAD signaling pathway. This is despite the observation that the SCC25 cells express very little type II receptor mRNA but similar amounts of type I receptor mRNA compared with human epidermal keratinocytes (**Fig 2**). These data suggested that (i) TGF-β1-induced growth inhibition in human epidermal keratinocytes was mediated by events downstream of TGF-β1-mediated *trans*-activation or (ii) that the signaling pathway mediating growth inhibition was independent of that required for SMAD-mediated *trans*-activation.

To examine the possibility of an alternative growth inhibitory pathway in human epidermal keratinocytes we assayed for the phosphorylation status of Rb in TGF-β1-treated human epidermal keratinocytes and SCC25 cells (**Fig 3*A–C***). TGF-β1 induced a time-dependent decrease in DNA synthesis in human epidermal keratinocytes (**Fig 3*A***) but not in SCC25 cells (**Fig 3*B***). TGF-β1 treatment also caused a marked reduction in both the hyperphosphorylated form of Rb and the cell cycle regulator, cdc2 (**Fig 3*C***). In contrast, the SCC25 cells (growth inhibitor insensitive) did not show any alteration in Rb phosphorylation status or cdc2 protein levels during a similar time period (**Fig 3*B***). These data confirmed that Rb hypophosphorylation and cdc2 downregulation were good markers of TGF-β1-mediated growth inhibition in keratinocytes. We now examined whether the regulation of Rb hypophosphorylation was similar to that for 3TP-Lux, i.e., was transcription dependent. We tested this possibility in human epidermal keratinocytes by examining the effects of a 12 h exposure to TGF-β1 on Rb phosphorylation status in the presence or absence of 250 ng per ml actinomycin D (Saunders *et al*, 1998). **Figure 4** demonstrates that the loss of phosphorylated Rb, in response to TGF-β1, is inhibited by actinomycin D. As actinomycin D alone did not significantly perturb the phosphorylation status of Rb these data suggested that TGF-β1-mediated human epidermal keratinocyte growth inhibition operates through a transcription-dependent signaling pathway. In this regard, the regulation of growth inhibition by TGF-β1 was similar to the regulation of 3TP-Lux activation.

TGF-β1 activates SMAD7-sensitive and SMAD7-insensitive pathways We now examined whether both growth inhibition and 3TP-Lux activation were SMAD-dependent events. SMAD2-, SMAD3-, and SMAD4-dependent signaling of 3TP-Lux can be inhibited by SMAD7 (Nakao *et al*, 1997; Itoh *et al*, 1998). Furthermore, SMAD7 has been shown to be induced in cells following TGF-β1 treatment (Afrakhte *et al*, 1998). Therefore, we examined the expression of SMAD7 following TGF-β1 treatment. Treatment of human epidermal keratinocytes with TGF-β1 (20 ng per ml) caused a rapid and time-dependent induction of SMAD7 mRNA that was maximal at 2 h (3.5-fold) and returned to basal levels by 4 h (**Fig 5*A***). The induction of SMAD7 by TGF-β1 suggests that SMAD7 is likely to be a negative regulator of SMAD signaling in keratinocytes. We therefore examined the effects of overexpressing SMAD7 on the ability of TGF-β1 (i) to induce PAI-1 promoter activity (estimated by 3TP-Lux activation), (ii) to inhibit the promoter activity of the proliferation-specific marker cdc2, or (iii) to induce the promoter activity of the growth-arrest-specific gene, p21 (Datto *et al*, 1995). It has been previously shown that cdc2 promoter activity is both proliferation specific (Saunders *et al*, 1993a; Saunders and Jetten, 1994; Dahler *et al*, 1998) and E2F:Rb regulated (Dalton, 1992; Tommasi and Pfeifer, 1995) whereas that of the p21 promoter is growth arrest specific and SMAD3, SMAD4, and Sp1 dependent (Datto *et al*, 1995; Li *et al*, 1998; Moustakas and Kardassis, 1998). It is also important to note that p21 inhibits Rb phosphorylation resulting in growth inhibition (Dyson, 1998). Treatment of keratinocytes with TGF-β1 resulted in a 6-fold increase in the activity of the 3TP-Lux reporter construct (**Fig 5*B***). This increase in 3TP-Lux activity was inhibited 3-fold by the cotransfection of a SMAD7 expression plasmid (**Fig 5*B***). Treatment of keratinocytes with TGF-β1 resulted in a 3-fold decrease in the activity of the cdc2 promoter (**Fig 5*C***). This is similar in magnitude to the decrease in cdc2 mRNA expression reported in keratinocytes following TGF-β1 treatment (Saunders and Jetten, 1994). Cotransfection of the SMAD7 expression

plasmid did not inhibit TGF-β1-mediated cdc2 suppression (**Fig 5*C***). These data indicate that the signaling pathway regulating the TGF-β1-mediated suppression of the proliferation-specific marker cdc2 is SMAD7 insensitive. Treatment of keratinocytes with TGF-β1 resulted in a 3.5-fold increase in the p21 promoter activity (**Fig 5*D***). The induction of p21 promoter activity by TGF-β1 was inhibited by cotransfection of SMAD7 (**Fig 5*D***). These data indicate that treatment of keratinocytes with TGF-β1 initiates events that lead to the suppression or induction of the critical cell cycle genes, cdc2 and p21, respectively. The data also show that this regulation is not mediated through the same signaling pathway, however.

Suppression of TG 1 mRNA expression is SMAD7 sensitive As the suppression of growth and the suppression of differentiation are functionally distinct outcomes of TGF-β1 action in keratinocytes we examined whether the suppression of differentiation by TGF-β1 was defective in the SCC25 cells. **Figure 6** demonstrates that TGF-β1 can inhibit IFN-γ- as well as TPA-mediated induction of TG 1 mRNA expression in keratinocytes (**Fig 6**). These data extend our earlier observation that TGF-β1 could also inhibit IFN-γ-induced expression of another differentiation-specific marker, cornifin (Saunders and Jetten, 1994). As TPA and IFN-γ actions are mediated by different signaling pathways this would suggest that differentiation suppression is a general property of TGF-β1 in keratinocytes. An examination of TG 1 mRNA levels in the SCC25 cell line revealed that, although neither TPA nor IFN-γ could induce TG 1 mRNA levels, the constitutive expression of TG 1 mRNA was suppressed by TGF-β1 in untreated as well as TPA- or IFN-γ-treated SCC25 cells (**Fig 6**). These data indicate that, unlike the growth inhibitory

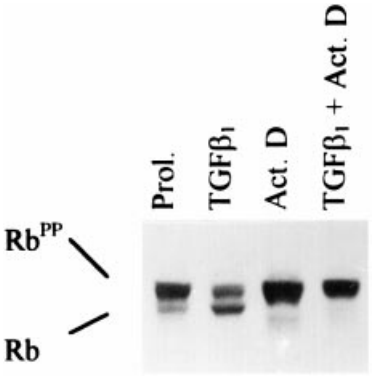
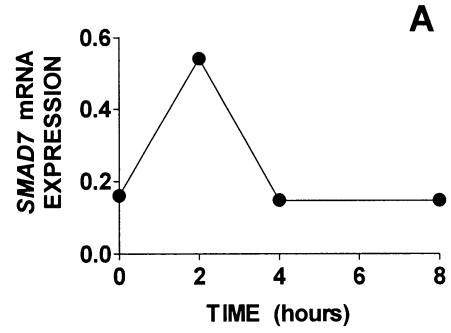
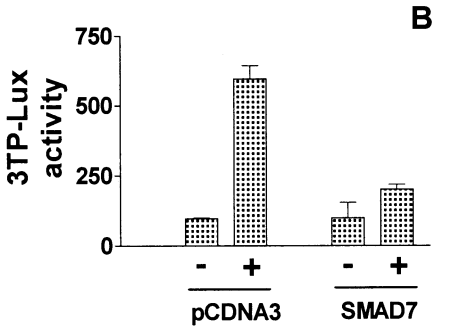
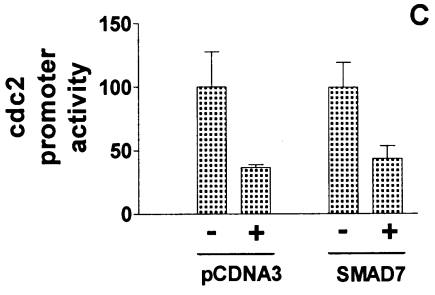


Figure 4. TGF-β1-induced hypophosphorylation of Rb is inhibited by actinomycin D. Proliferating human epidermal keratinocytes were left untreated (Prol.) or were treated for 12 h with 20 ng per ml TGF-β1 (TGF-β1), 250 ng per ml actinomycin D (Act. D), or TGF-β1 + actinomycin D (TGF-β1 + Act. D). The hyphosphorylated (Rb) and hyperphosphorylated (Rb^{PP}) forms of Rb were determined as described in **Fig 3**.







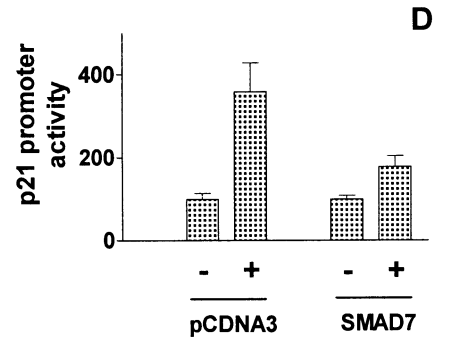


Figure 5. SMAD7 inhibits 3TP-Lux activation and p21 promoter activation but not cdc2 promoter suppression. (*A*) Total RNA was harvested from human epidermal keratinocytes at various times following TGF-β1 treatment. Twenty micrograms of total RNA was then electrophoresed and transferred onto nylon membrane. The expression of SMAD7 and GAPDH mRNA was detected using specific [^{32}P]-labeled cDNA probes followed by phosphorimage analysis. Data are expressed as arbitrary expression levels normalized for GAPDH expression. (*B*) Proliferating human epidermal keratinocytes were transfected with both 0.3 μg β-actin CAT and 1 μg of 3TP-Lux plus either 0.3 μg of SMAD7 expression vector or the control vector pCDNA3. Cells were then treated with (+) or without (–) 20 ng per ml TGF-β1 for 48 h. 3TP-Lux was then normalized for transfection efficiency against the β-actin CAT activity and the data were plotted as a percentage of the value obtained in the absence of TGF-β1. (*C*) Human epidermal keratinocytes were treated the same as for (*B*) with the exception that the cells were transfected with 1 μg of cdc2-CAT reporter and 0.3 μg of β-actin Luc. The ratio of reporter to SMAD7 expression plasmid was the same as in (*B*). (*D*) Human epidermal keratinocytes were treated the same as for (*B*) with the exception that the cells were transfected with 1 μg of p21 luciferase reporter and 0.3 μg of β-actin CAT. The ratio of reporter to SMAD7 expression plasmid was the same as in (*B*). All data (except *A*) are presented as mean ± SEM of triplicate determinations from three experiments.

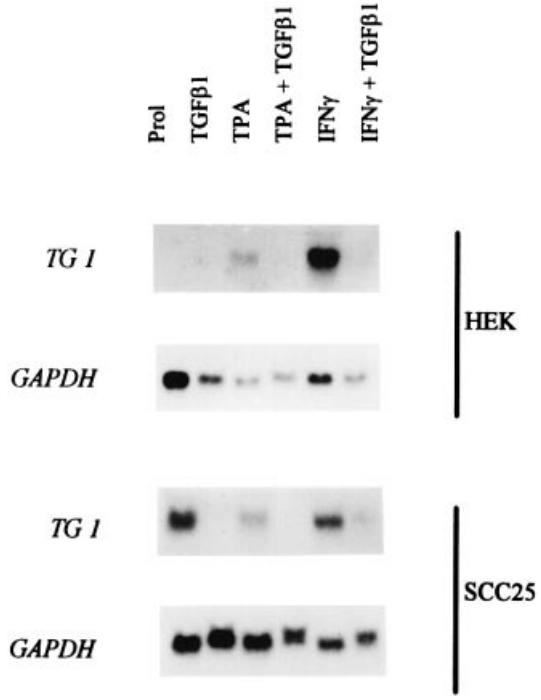


Figure 6. Suppression of TG 1 mRNA expression by TGF-β1 occurs in both human epidermal keratinocytes and SCC25 cells. Human epidermal keratinocytes or SCC25 cells were either left untreated (Prol.) or treated for 48 h with TGF-β1 (20 ng per ml), TPA (50 ng per ml), IFN-γ (100 U per ml), or a combination of TPA + TGF-β1 or IFN-γ + TGF-β1. Following incubations, total RNA was harvested and 20 μg fractionated by electrophoresis and transferred to nylon membrane. Membranes were then probed for expression of either TG 1 or GAPDH.

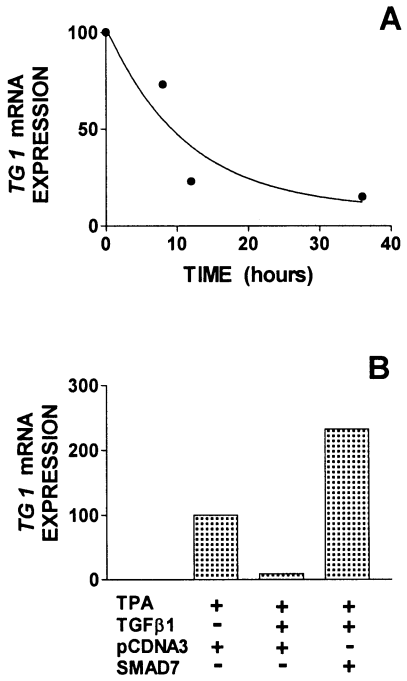


Figure 7. Time-dependent suppression of TG 1 by TGF-β1. (*A*) Expression of TG 1 in human epidermal keratinocytes that have been treated with 50 ng per ml TPA for 24 h to induce differentiation followed by treatment with TPA + TGF-β1 (20 ng per ml) for various times. Total RNA was isolated and 20 μg subjected to northern blotting and probed for TG 1 and GAPDH expression. Expression was quantitated by densitometry and TG 1 expression was normalized for GAPDH expression to that of GAPDH by densitometry. (*B*) Human epidermal keratinocytes were induced to differentiate by treatment with TPA (TPA; 50 ng per ml) for 16 h. Cells were then transfected with expression vectors encoding nothing (pCDNA3) or SMAD7 (SMAD7) and treated with TGF-β1 (TGF-β1; 20 ng per ml) for 16 h. All cells were cotransfected with a GFP-expressing plasmid. Transfected cells expressing GFP were then FACS sorted and the expression of TG 1 and actin mRNA was determined by RT-PCR in the transfected human epidermal keratinocytes. The expression of TG 1 mRNA is presented as a percentage of the TG 1 mRNA expression in the TPA differentiated keratinocytes. TG 1 mRNA expression levels have been normalized for actin mRNA expression.

pathways, the signaling pathway regulating differentiation suppression is intact in the SCC25 cells. This is similar to the presence of an intact pathway regulating PAI-1 promoter activity (estimated by 3TP-Lux) in the SCC25 cells.

The mechanism by which TGF-β1 decreases TG 1 mRNA levels is currently unknown. Consequently, we examined whether the TGF-β1-mediated suppression of TG 1 mRNA was SMAD7 inhibitable. In the first instance, we examined a time course for the suppression of TG 1 mRNA in human epidermal keratinocytes treated with TPA for 36 h (induces differentiation) and then cotreated with TGF-β1 for varying times to 36 h (**Fig 7*A***). This experiment indicated that the TG 1 mRNA was rapidly decreased in the presence of TGF-β1 ($t_{1/2} = 7.9$ h). We next examined whether the suppression of differentiation, as characterized by TG 1 mRNA expression, by TGF-β1 in keratinocytes was SMAD7 inhibitable. To do this, human epidermal keratinocytes were treated with the differentiation-inducing agent TPA (50 ng per ml) for 16 h to induce TG 1 mRNA (Dicker *et al*, in press). Following this cells were cotransfected with either the pCDNA3 vector or the SMAD7 expression vector plus CMV-GFP. We had previously established that under these conditions SMAD7 is able to inhibit TGF-β1-mediated p21 and 3TP-Lux induction (**Fig 5**). These cells were then treated with TPA (50 ng per ml) or TPA (50 ng per ml) + TGF-β1 (20 ng per ml) for a further 16 h. Cells were then harvested and the transfected cells (GFP expressing) were sorted by FACS (approximately 3.5%–5% transfection efficiency). RNA was then isolated from the FACS cells and the expression of TG 1 and actin mRNA was determined by RT-PCR analysis. **Figure 7(*B*)** clearly shows that TGF-β1 suppresses TG 1 mRNA expression by 90% whereas overexpression of SMAD7 can prevent this suppression and in fact leads to 2-fold greater levels of expression of TG 1 mRNA. These data indicate that TGF-β1-mediated TG 1 suppression is SMAD7 inhibitable.

DISCUSSION

In this study we have examined the signaling pathways involved in the regulation of markers of differentiation, ECM production, and proliferation in response to TGF-β1 in primary cultures of human keratinocytes. Our data suggest that multiple TGF-β signaling pathways exist in keratinocytes and that these may be linked to the regulation of different biologic functions (**Fig 8**). In this study we found that TGF-β1-mediated growth inhibition of human epidermal keratinocytes was associated with two functionally distinct pathways. In the first instance TGF-β1 induced the expression of the established growth suppressor, p21 (Datto *et al*, 1995). The induction of p21 was mediated by a SMAD7-inhibitable pathway, which is consistent with reports that p21 induction is mediated by SMAD3, SMAD4 interacting with the transcription factor Sp1 in the p21 promoter (Moustakas and Kardassis, 1998). In contrast, TGF-β1 also suppressed the activity of the cdc2 promoter. cdc2 is also a well-established cell cycle regulator that is essential for cell division (Lee and Nurse, 1987; Heuvel and Harlow, 1993; Dahler *et al*, 1998). The inability of SMAD7 to inhibit TGF-β1-mediated suppression of cdc2 promoter activity occurred at a concentration of SMAD7 that we had shown could inhibit 3TP-Lux activation, TG 1 suppression, and p21 induction. These data suggest that TGF-β1 induces growth arrest by initiating a cascade of events (both SMAD7-

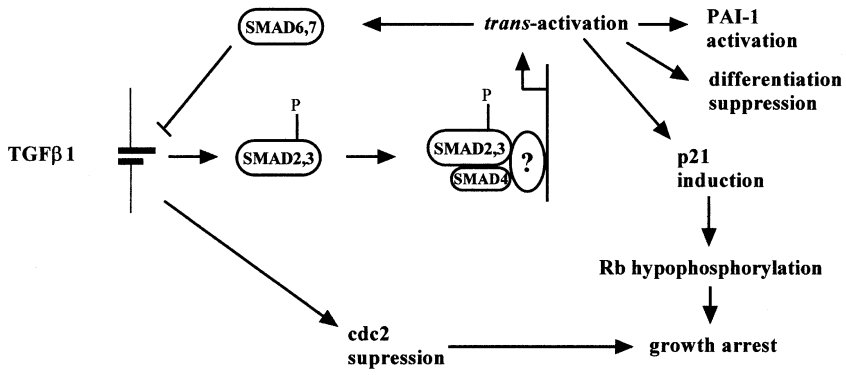


Figure 8. Proposed signaling pathway mediating TGF-β1 actions in human keratinocytes. The proposed pathway links TGF-β1-mediated suppression of differentiation and induction of PAI-1 and p21 to the SMAD7-inhibitable pathway. In contrast, the inhibition of cdc2 is hypothesized to be mediated by a ligand/receptor-dependent but SMAD7-insensitive pathway.

sensitive and SMAD7-insensitive) that leads to the suppression of the cell cycle. Evidence of multiple signaling pathways involved in TGF-β1-induced growth suppression is also supported by our observations that both the human epidermal keratinocytes and SCC25 cells could regulate TG 1 suppression and 3TP-Lux induction in response to TGF-β1 whereas the SCC25 cells were not growth inhibited by TGF-β1 and did not accumulate hypophosphorylated Rb in response to TGF-β1. The inability of TGF-β1 to inhibit growth of SCC25 cells occurred despite the fact that they express p21 protein but not p53 (unpublished observations). Moreover, the SCC25 cells did not express detectable levels of type II TGF-β receptor mRNA, which has previously been suggested to regulate growth inhibitory responses to TGF-β1 (McCaffrey *et al*, 1995). In addition, the dose–response profile for the activation of the 3TP-Lux in human epidermal keratinocytes revealed an Ec_{50} value (0.41 ± 0.16 ng per ml) that was almost an order of magnitude less than that for growth suppression (3.2 ± 1.3 ng per ml). These observations strongly implicate a complex signaling pathway involved in growth inhibition.

The mechanism by which TGF-β1 mediates growth suppression in human epidermal keratinocytes was not addressed in this study as it has been the subject of extensive work previously (Hannon and Beach, 1994; Datto *et al*, 1995; Li *et al*, 1995; Reynisdottir *et al*, 1995; Iavarone and Massague, 1997; Nagahara *et al*, 1999). Our data clearly demonstrate, however, that TGF-β1-mediated growth suppression is associated with a suppression of cdc2 protein levels and promoter activity, an induction of p21 promoter activity, and the accumulation of hypophosphorylated Rb. Rb is a known target of TGF-β1 action and is also a key regulator of E2F-mediated G1/S phase transition (Dyson, 1998). Furthermore, previous studies have shown that TGF-β1 can induce p21 (Datto *et al*, 1995) and p15 (Hannon and Beach, 1994; Li *et al*, 1995) and repress cdc25a (Iavarone *et al*, 1997) or cyclinE:cdk2 activity (Nagahara *et al*, 1999), all of which ultimately affect E2F activity. Thus, our data are consistent with earlier reports in which Rb and E2F activity were the downstream effectors of TGF-β-mediated growth inhibition. Our study extends these findings, however, and suggests that a SMAD7-independent pathway may also be invoked during TGF-β1-mediated growth suppression. This pathway is characterized by the downregulation of cdc2 in response to TGF-β1. Although the signaling events mediating cdc2 suppression are unknown it is clear that this process is not downstream of the cell cycle arrest caused by p21 induction and Rb hypophosphorylation as cdc2 suppression is SMAD7 insensitive whereas that of p21 induction is SMAD7 inhibitable.

The inability of SMAD7 to inhibit TGF-β1-mediated cdc2 suppression as well as the inability of SCC25 cells to undergo growth arrest in response to TGF-β1 suggests that the transcriptional suppression of cdc2 may be regulated by different domains on the type I or II TGF-β receptors to those mediating SMAD-dependent 3TP-Lux activation, p21 induction, or TG 1 suppression. This has not been shown in keratinocytes previously but is consistent with earlier data in which growth suppression and PAI-1 activation were dissociated in cells in which the type II receptor was truncated or poorly expressed (Chen *et al*, 1993; McCaffrey *et al*, 1995). Our finding of negligible expression of the type II receptor mRNA in the SCC25 cells is consistent with this view and may indicate a role for the type II receptor in TGF-β1-mediated cdc2 suppression in keratinocytes. Further evidence supporting an independent role for the type II receptor in TGF-β signaling comes from earlier reports in which SMAD7 inhibited PAI-1 activation by inhibiting the binding of SMAD2 or SMAD3 to the type I receptor (Hayashi *et al*, 1997; Nakao *et al*, 1997). Thus, our data suggest that suppression of cdc2 is mediated by a pathway requiring a different domain on the type I receptor to that required for PAI-1 activation or that the signaling pathway is initiated from the type II receptor.

It has been reported previously that TGF-β1-mediated growth inhibition of B cells by activin (Ishisaki *et al*, 1998) and MvLu1 cells by TGF-β1 (Itoh *et al*, 1998) is attenuated (not abolished) by SMAD7. Although we did not specifically examine the effects of SMAD7 on TGF-β1-mediated growth suppression in this study we did show that TGF-β1-mediated growth inhibition involves both induction of a growth inhibitor, p21, as well as suppression of a proliferation-specific gene, cdc2. Both of these TGF-β1-dependent events would be expected to be growth inhibitory. Thus, the inability of SMAD7 to totally abolish TGF-β1-mediated growth suppression in earlier studies (Ishisaki *et al*, 1998; Itoh *et al*, 1998) may be due to the residual growth inhibitory activity mediated by a SMAD7-independent pathway. Although cdc2 has been reported to be involved in S phase entry (Itzhaki *et al*, 1997) it is primarily thought to be involved in a G2/M phase arrest. TGF-β1 treatment is generally associated with a G1 arrest (Nagahara *et al*, 1999) although it has also been reported to induce a G2 arrest in keratinocytes (Shipley *et al*, 1986). The exact mechanism of the TGF-β1-induced G2 arrest in keratinocytes is unclear but is consistent with our findings. It is interesting to note that a recent report suggested that cyclinB:cdc2 could bind to the type II TGF-β receptor and was inactivated by TGF-β treatment (Liu *et al*, 1999). Although it is unknown whether this interaction occurs in keratinocytes it is consistent with the finding that the SCC25 cells do not undergo growth arrest in response to TGF-β1 and do not express type II receptor mRNA. Regardless of the role of cdc2 suppression in TGF-β1-mediated actions our data clearly show (i) that TGF-β1 induces a transcriptional suppression of cdc2 and (ii) that this suppression is mediated by an unknown and SMAD7-insensitive signaling pathway.

Alternatively, the SMAD7-insensitive pathway of cdc2 suppression may play an important role in other TGF-β1-mediated actions such as the maintenance of genome stability (Glick *et al*, 1999). TGF-β1-induced stabilization of the genome was reported to occur at a dose of TGF-β1 that was lower than that required for growth suppression. Such a finding is consistent with the existence of multiple TGF-β1 signaling pathways in keratinocytes. It would be interesting to examine whether this pathway is SMAD7 sensitive or not.

We had previously reported that TGF-β1 was able to inhibit IFN-γ-induced expression of the squamous differentiation-specific markers TG 1 and cornifin (Saunders and Jetten, 1994). In this study we extend these findings to show that TGF-β1 can suppress the expression of the differentiation-specific marker TG 1 following either TPA- or IFN-γ-induced differentiation in keratinocytes. Furthermore, we show that the constitutive expression of TG 1 by SCC25 cells can also be suppressed by TGF-β1. This suppression is presumed to be transcriptional as we were unable to show any alteration in TG 1 mRNA stability in response to TGF-β1 (data not shown). It remains unclear, however, whether the suppression of TG 1 is mediated directly by SMAD transcription factors, as it is for PAI-1 induction, or whether TG 1 suppression is mediated by events downstream of SMAD-dependent transcriptional activation. It is of interest to note that a reporter construct containing the proximal 2.9 kb of the transglutaminase type 1 promoter could not be suppressed by TGF-β1 in differentiated keratinocytes (data not shown), although this region of the promoter was recently shown to contain sufficient elements to direct transcription in a tissue-specific and differentiation-specific manner in transgenic mice (Medvedev *et al*, 1999). These data would suggest that the TGF-β1-rsponsive elements must lie outside this 2.9 kb region. Notwithstanding this, our data clearly show that proximate signaling events controlling PAI-1 induction and TG 1 suppression are similar as they are both inhibited by SMAD7.

An examination of the signaling pathways responsible for TGF-β1 actions in normal keratinocytes provides insight into the role of TGF-β1 in neoplastic progression. For instance, recent studies (Cui *et al*, 1996; Portella *et al*, 1998) have demonstrated that during neoplasia the disruption of TGF-β1 actions occurs in two distinct phases. In the first instance, the loss of TGF-β1-mediated growth suppression occurs. In contrast, the second phase of transformation to a malignant spindle cell carcinoma is actually induced by TGF-β1 (Cui *et al*, 1996). These apparently contradictory findings may reflect the presence of defects at different points downstream of TGF-β1 signaling that are associated with different functional outcomes or signaling pathways.

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